Protein Kinase C-mediated Phosphorylation of Troponin I and C-protein in Isolated Myocardial Cells Is Associated with Inhibition of Myofibrillar Actomyosin MgATPase*

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Richard C. Venema and J. F. Kuo
From the Department of Pharmacology, Emory University School of Medicine, Atlanta, Georgia 30322

Phosphorylation of cardiac myofilibrillar proteins by protein kinase C (PKC) in isolated adult rat cardiomyocytes has been compared with that mediated by the cAMP-dependent protein kinase (PKA). PKA activation by β-adrenergoreceptor (isoproterenol) stimulation results in stoichiometric phosphorylation of troponin I (TnI) and C-protein. PKC activation by either 12-O-tetradecanoylphorbol-13-acetate (TPA) or by α-adrenergoreceptor (phenylephrine plus propranolol) stimulation results in phosphorylation of the same two proteins to similar extents. Two-dimensional phosphopeptide mapping shows that the same sites in TnI are modified by PKC in vitro and in TPA- or α-agonist-stimulated cells. These sites are distinct from those phosphorylated in isoproterenol-stimulated cells or by PKA in vitro. Phosphopeptide mapping analysis of C-protein shows that PKC and PKA phosphorylate identical residues in this protein in vitro and in situ. TPA-stimulated phosphorylation in myocytes is associated with a reduction in maximal activity of myofibrillar Ca2+-dependent actomyosin MgATPase. Isoproterenol-stimulated phosphorylation has no effect on maximal activity but reduces the Ca2+ sensitivity of the MgATPase. These data demonstrate that TnI and C-protein are phosphorylated in myocardial cells by both PKA and PKC, resulting in different functional consequences in each case.

Over the past 20 years, many laboratories have focused their investigation on the β-adrenergic regulation of cardiac contractility through phosphorylation of cellular substrates by the cAMP-dependent protein kinase (PKA)† (1, 2). Based on these studies, there is now general agreement that PKA-mediated protein phosphorylation has an important role in myocardial physiology. Stimulation of cardiac myocytes (the characteristic cell type of the heart) by catecholamines or β-adrenergic agonists (i.e. isoproterenol) produces an elevation of intracellular Ca2+. This occurs primarily through PKA-mediated phosphorylation and activation of sarcoplasmatic Ca2+ channels (3) and the sarcoplasmic reticulum Ca2+ pump regulatory protein, phospholamban (4). The resulting increased availability of Ca2+ to the contractile apparatus causes an increase in the force of cardiac contraction (positive inotropic effect). Direct phosphorylation of the contractile machinery itself is also recognized as having an important modulatory role in contraction (1, 2). Considerable effort, therefore, has gone into the study of PKA-catalyzed phosphorylation of cardiac contractile proteins and its functional consequences in mammalian heart cells. It is now well established that β-adrenergic stimulation of either intact hearts or isolated myocardial cells results in phosphorylation of C-protein and troponin I (TnI) in the thick and thin filaments, respectively, of cardiac myofilaments (1, 2, 5–9).

Cardiac myofilaments, like those of other muscle cell types, contain an intrinsic actomyosin MgATPase activity, the molecular basis of contraction. Sensitivity of the actomyosin MgATPase to Ca2+ in striated muscle is mediated by the troponin complex in the thin filament. This multiprotein complex is comprised of the Ca2+-binding TnC subunit, the MgATPase-inhibiting TnI subunit, and the tropomyosin-binding TnT subunit. Phosphorylation of TnI by PKA results in a decreased myofibrilar Ca2+ sensitivity (8–10) because of a reduced affinity of TnC for Ca2+ (11). Decreased Ca2+ responsiveness may function as a negative feedback mechanism or contribute to the increased relaxation rate of beating hearts under the influence of β-adrenergic stimulation (12). The function of C-protein in cardiac muscle is currently unknown as are the consequences, if any, of its phosphorylation. Phosphorylation of C-protein does not appear, for example, to have a role in modulating myofibrilar Ca2+ sensitivity in mammalian heart cells (9).

Another important signaling protein kinase, protein kinase C (PKC) (13), also has a role in cardiac physiology. Although its precise function is not yet clear, several lines of evidence implicate this protein kinase in cardiac regulation. A number of receptor systems in heart cells are coupled to PKC activation, including the muscarinic cholinergic (14), angiotensin II (15), and α-adrenergic (14, 16–19) receptor systems. Furthermore, phorbol esters (activators of cellular PKC) are known to have a pronounced negative inotropic effect on a variety of cardiac preparations, including the single cardiomyocyte (15, 20, 21). It is assumed that the inhibitory effect is mediated via PKC-catalyzed protein phosphorylation. Very little is known, however, with regard to which myocardial substrates are important in the contractile response. Also unclear is whether the contractile machinery is affected directly or whether it is only indirectly affected through changes in Ca2+ homeostasis. However, PKC target substrates are likely to be found in the myofilament compartment of heart cells since the enzyme is translocated to the myofibrils upon α-adrenergic stimulation (17) and has been immunocytochemically
localized at these sites in unstimulated myocytes (22). Moreover, several myocardial contractile proteins are stoichiometrically phosphorylated by PKC in vitro including C-protein (23), TnI, and TnT (24–26). Phosphorylation of TnI or TnT by PKC in the troponin or troponin-tropomyosin complex results in a decreased Ca^{2+}-dependent MgATPase activity in reconstituted actomyosin (26). Whether this mechanism is involved in the negative inotropic effect of phorbol esters on intact myocardial cells is presently unclear. Also, the question of whether these or other myofibril proteins are phosphorylated by PKC in adult heart cells has not been definitively answered.

Previous investigation in this laboratory has provided evidence that TnT and, to a lesser extent, TnI are phosphorylated in neonatal rat cardiomyocytes upon activation of PKC by the phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA) (22). Determination of the stoichiometry, site specificity, and functional consequences of these phosphorylation reactions is now required to assess their physiological significance. Furthermore, it is important to determine whether these reactions occur in adult heart cells since neonatal and adult mammalian hearts express different isoforms of both TnI (27) and TnT (28). Cloning and sequencing of rat neonatal TnI has revealed that it lacks the preferred phosphorylation sites for both PKA- and PKC-mediated phosphorylation (27). This may explain (at least in part) the relative insensitivity of the immature myocardium to the inotropic effects of both α- and β-adrenergic agonists (29). Finally, further investigation of PKC effects in adult heart cells is warranted because, in disagreement with our published results with neonatal myocytes (22), other studies have concluded that PKC does not phosphorylate cardiac myofibrillar proteins in adult mammalian hearts (30, 31). In the present study, therefore, we have examined and compared TPA-, α-adrenergic-, and β-adrenergic-stimulated phosphorylation of myofibrillar proteins in isolated adult rat cardiomyocytes. Phosphorylation of native myofibrils by PKC and PKA in vitro has also been investigated. PKC-mediated phosphorylation has been compared with PKA-mediated phosphorylation in terms of its differential substrate specificity, stoichiometry, site specificity, and functional consequences.

**EXPERIMENTAL PROCEDURES**

**Materials**—Collagenase B was purchased from Boehringer Mannheim; phosphate-free Dulbecco’s minimum essential medium was from Gibco; calcineurin A and TPA were from LC Services Corp.; forskolin, dibutyryl cAMP, (−)isoproterenol HCl, phenylephrine HCl, propanolol HC1, tetrodotoxin (diphenylcarbamoyl chloride-treated), phosphatase b, phosphatase kinase, phosphatidylinositol, diol/en, cAMP, 3-isotouyl-1-methylxanthine, fosphone/un, and calmodulin were from Sigma; [32P]orthophosphate and [γ-32P]ATP were from ICN Radiochemicals. Cardiac myosin light chain 2, myosin light chain 1, and myosin light chain 3 (MLCP) were from Sigma. 

**Experimental Procedures**

**Phosphorylation of Myofibrils in Intact Cardiomyocytes with [32P]Orthophosphate**—Isolated myofibrils from one rat heart were suspended in 10 ml of phosphate-free Dulbecco’s minimum essential medium containing 10 mM orthophosphate (1.0 μCi/ml). Cells were incubated in a culture flask for 2 h at 37 °C in a 5% CO2 humidified incubator. After 2 h, aliquots (1 ml) of [32P]labeled cell suspension were pipetted into Control, 100 nM TPA, 100 nM isoproterenol, 50 μM phenylephrine plus 1 μM propanolol, or 100 nM calcineurin A (in dimethyl sulfoxide, 10 μl) was added, and further incubation at 37 °C was carried out for 10 min. Control incubations were performed by adding dimethyl sulfoxide (solvent control) only. At the end of the incubation period, myocyte pellets were then homogenized in ice cold “inhibiting” buffer (50 μl, modified to contain 1% Triton X-100 plus the protease and phosphatase inhibitors indicated above, and left on ice for 30 min. Detergent-extracted myofibrils were pelleted at 5,000 × g. Myofibril proteins were then immediately solubilized in sodium dodecyl sulfate (SDS) sample buffer by heating for 5 min at 100 °C.

**Phosphorylation of Isolated Myofibrils by PKC and PKA**—Detergent-extracted cardiac myofibrils were prepared from previously frozen adult rat hearts by the method described by Stull and Buss (33) for preparation of myofibrils from beef hearts. The protease inhibitors indicated above (but not the phosphatase inhibitors) were included in all incubation buffers. PKC was purified from bovine brain through the phenyl-Sepharose step as described previously (34). Bovine brain PKA was purified according to the method of Miyamoto et al. (35). Isolated myofibrils were phosphorylated by PKC in the presence and absence of phospholipids, diol/en, and Ca^{2+} under conditions described previously (36), modified to contain 1 μM of PKC, TPA, and TPA plus at high concentrations of ATP, and TNP phosphorylase-Isolated myofibrils were prepared from one rat heart (31, 32). Myofibrils were then immediately solubilized in sodium dodecyl sulfate (SDS) sample buffer by heating for 5 min at 100 °C.

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to x-ray film for equal lengths of time. Proteins were phosphorylated in vitro with a higher specific radioactivity of ATP and, therefore, were exposed to film for shorter times. Two-dimensional phosphoamino acid analysis was performed according to Cooper et al. (40). Hydrolyzed samples were electrophoresed for 45 min at 1,250 V in pyridine:acetic acid:water (0.5:5:94.5, v/v) followed by ascending chromatography in isobutyric acid, 0.5 M ammonium hydroxide (5:3, v/v). Phosphoamino acids were visualized by ninhydrin spraying and autoradiography.

Actomyosin MgATPase Assay—The effects of TPA and isoproterenol on myofibrillar actomyosin MgATPase activity were determined in experiments similar to those used to analyze phosphorylation. Myocytes from three rat hearts were pooled and incubated in 30 ml of Dulbecco's minimum essential medium for 2 h. The cell suspension was divided into 3 aliquots of 10 ml each and incubated for a further 10 min with either no addition (control), 100 nM TPA, or 100 nM isoproterenol. Myofibrils were then prepared as described by Murphy and Solaro (41) except that 100 nM calyculin A was included in all isolation buffers to prevent dephosphorylation. Protein concentrations of the myofibrillar preparations were determined by the method of Bradford (39). Ca²⁺-dependent actomyosin MgATPase activities of equal quantities (60 µg) of myofibrillar protein from each condition were measured at different concentrations of free Ca²⁺, varied through the use of Ca²⁺/EGTA buffers (26).

RESULTS

Phosphorylation of Cardiac Myofibrils by PKC and PKA in Vitro—Isolated cardiac myofibrils from adult rat heart contained proteins identified by Coomassie staining of SDS-polyacrylamide gels as myosin heavy chain (205 kDa), C-protein (150 kDa), actin (45 kDa), TnT (43 kDa), tropomyosin (35 kDa), TnI (30 kDa), myosin light chain 1 (MLC1) (27 kDa), and myosin light chain 2 (MLC2) (19 kDa) (Fig. 1). Myofibrils were examined as substrate for PKC and PKA in vitro (Fig. 1 and Table I). The PKA holoenzyme selectively phosphorylated C-protein and TnI in myofibrils in a CAMP-dependent manner. A third phosphorylated protein visible on autoradiograms represented autophosphorylation of the PKA regulatory subunit. Phosphorylation of the two myofibrillar proteins was maximal after a 60-min incubation at 30 °C and reached a stoichiometry of 1.5 and 0.9 mol of phosphate/mol of C-protein and TnI, respectively. Phosphorylation of intact myofibrils by PKC in vitro was more extensive than that by PKA. PKC incorporated phosphate stoichiometrically into four myofibrillar proteins identified as C-protein (1.5 mol/mol), TnT (1.3 mol/mol), TnI (0.8 mol/mol), and MLC2 (0.6 mol/mol). Phosphorylation was maximal within 60 min and was completely dependent on the presence of phospholipid, diolein, and Ca²⁺.

Phosphorylation of Cardiac Myofibrils by PKC and PKA in Cardiomyocytes—The PKC- and PKA-mediated phosphorylation of cardiac myofibrillar proteins was also investigated in isolated adult rat cardiomyocytes (Fig. 2 and Table I). Myofibrils prepared from ³²P-labeled control myocytes contained phosphate label in five different myofibrillar proteins identified by Coomassie staining (data not shown) as C-protein, TnT, troponin, TnI, and MLC2. The results shown are representative of at least 12 experiments.

### Table I

<table>
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![Fig. 1. Phosphorylation of isolated adult rat cardiac myofibrils in vitro by PKC and PKA. Myofibrils were incubated with [γ-³²P]ATP and either PKC or PKA for 60 min at 30 °C. Incubations with PKC were in the absence or presence of phosphatidylserine/diolen/Ca²⁺ (PS/DD/Ca²⁺) followed, and incubations with PKA were in the absence or presence of 20 µM cAMP. Phosphorylated proteins were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. Major phosphorylated proteins were identified by Coomassie staining as myosin heavy chain (MHC), C-protein (C-prot), actin, TnT, tropomyosin, TnI, MLC1, and MLC2. The results shown are representative of at least three experiments.](image_url)
those modified in situ. Activation of PKA in myocytes by isoproterenol treatment (100 nM for 10 min) resulted in the stoichiometric phosphorylation of two proteins in isolated myofibrils, C-protein (1.3 mol/mol) and TnI (1.0 mol/mol). In addition, a small increase in phosphate content of MLC2 (0.2 mol/mol) was consistently observed. PKA activation by forskolin (20 μM), dibutyryl cAMP (1 mM), or 3-isobutyl-1-methylxanthine (100 μM) gave qualitatively and quantitatively similar results (data not shown). The effect of TPA (100 nM for 10 min) on the state of phosphorylation of isolated myofibrils was also strikingly similar to that of isoproterenol in terms of its substrate specificity and phosphorylation stoichiometry. Activation of endogenous PKC by the phorbol ester resulted in stoichiometric phosphorylation of both C-protein (1.1 mol/mol) and TnI (0.8 mol/mol). Also, as observed with β- adrenergic stimulation, MLC2 was phosphorylated to a small extent (0.2 mol/mol) in response to TPA. In addition to the experiments in which 32P-labeled myofibrils were isolated and analyzed, other experiments were carried out in which 32P-labeled myocytes were incubated for 10 min with either no addition (control), 100 nM TPA, or 100 nM isoproterenol and then directly solubilized in SDS sample buffer prior to analysis of proteins by gel electrophoresis and autoradiography. The purpose of these experiments was to determine whether the phosphorylation state of isolated myofibril preparations accurately reflected that of the intact myocyte. Direct solubilization excludes the involvement of phosphorylation/dephosphorylation events that might occur during the washing, homogenization, and extraction procedures used to isolate myofibrils. The results of direct solubilization experiments were very similar to those obtained with detergent-extracted myofibrils except for a higher background of unidentified 32P-labeled proteins on gels (data not shown). The dose dependence and time dependence of TPA- and isoproterenol-stimulated phosphorylation were also investigated. TPA-stimulated phosphorylation was detected at 1 nM and was maximal at 10 nM. Isoproterenol effects were detected at 1 nM and were maximal at 100 nM. Phosphorylation in response to isoproterenol reached its maximum within 2 min of exposure to the agonist. In contrast, TPA stimulation required 10 min to reach a maximum.

Two-dimensional Phosphopeptide Mapping of TnI and C-protein Phosphorylated in Situ and in Vitro—The site specificity of phosphorylation of TnI in response to TPA- and β-adrenergic stimulation was examined by two-dimensional tryptic phosphopeptide mapping. A pattern of six tryptic phosphopeptides was obtained upon mapping of TnI phosphorylated under basal (control) conditions in myocytes (Fig. 3A). The number of tryptic phosphopeptides may be greater than the number of phosphorylation sites because of alternate cleavages by trypsin adjacent to certain charged amino acid or phosphoserine residues. TPA treatment resulted in an increase in 32P content of three phosphopeptides (spots 5, 6, and 8) and the appearance of a new phosphopeptide (spot 7) (Fig. 3B). Isoproterenol treatment stimulated phosphorylation of clearly different sites in TnI, as revealed by a very different pattern of phosphorylated peptides (Fig. 3C). No increase in labeling of phosphopeptides 5, 6, and 8 was observed in this condition. Instead, one very large and diffuse spot appeared (labeled as 9 on the map). Poor resolution of this spot may relate to the fact that PKA phosphorylates two adjacent residues (Ser-22 and Ser-23) in adult rat cardiac TnI (27). The large spot on the map probably represents a mixture of monophosphorylated and biphosphorylated derivatives of the same tryptic peptide. Sites in TnI that were phosphorylated in situ in response to TPA (Fig. 3B) (phosphopeptides 5, 6, 7, and 8) were also modified in vitro by PKC (Fig. 3E), indicating that direct phosphorylation by PKC occurs in myocytes, as opposed to indirect phosphorylation by a “downstream” kinase (42). Furthermore, the sites in TnI that were phosphorylated in situ in response to isoproterenol (Fig. 3C) (phosphopeptide 9) were also modified in vitro by PKA (Fig. 3F). Phosphopeptide mapping also revealed that minor amounts of nonspecific phosphorylation occurred in vitro (for example, spot 10 in the case of PKC and spots 5, 6, 7, and 8 in the case of PKA). Interestingly, nonspecific phosphorylation by PKA in vitro occurred at sites that were phosphorylated only by PKC (and not by PKA) in intact cells. Phosphopeptide mapping was further utilized to investigate the site-specific phosphorylation of TnI in cardiomyocytes treated with calyculin A, a highly potent inhibitor of phosphoprotein phosphatases 1 and 2A (43). Calyculin A (100 nM) stimulated an increase in the TnI phosphate content of 0.9 mol/mol after only 2 min of exposure to the inhibitor (data not shown). Incorporation of phosphate in the presence of calyculin A occurred at both PKC and PKA sites (Fig. 3D), indicating a rapid turnover of phosphate in these sites even in the absence of hormonal stimulation. One additional phosphopeptide was detected with calyculin A that was not detected with either TPA or isoproterenol (spot 3). This suggests that a third protein kinase may exist which also phosphorylates TnI in myocytes.

C-protein phosphorylation was also analyzed by two-dimensional tryptic phosphopeptide mapping. A single tryptic phosphopeptide (spot 5) was obtained for C-protein phosphorylated in control myocytes (Fig. 4A). Mapping of C-protein from isoproterenol-stimulated cells showed an increase in
Cardiac Myofibrillar Protein Phosphorylation by PKC and PKA

Myofibrils were phosphorylated in situ in 32P-labeled cardiomyocytes by incubation for 2 h with 32P, followed by a further 10-min incubation with either no addition (control), 100 nM TPA, 100 nM isoproterenol (Isop.), or 100 nM calyculin A (CL-A). Myofibrils were also phosphorylated in vitro by incubation with [γ-32P]ATP and either PKC or PKA. Equal amounts of myofibrillar protein from each condition were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. C-protein was excised from the gel, trypsin digested, and subjected to two-dimensional tryptic phosphopeptide mapping. The origins are indicated by arrows. The radioactivity in each sample was: A, control (456 cpm); B, TPA (1,530 cpm); C, isoproterenol (1,690 cpm); D, calyculin A (487 cpm); E, PKC in vitro (3,360 cpm); F, PKA in vitro (3,492 cpm). Similar results have been obtained in four separate experiments.

Additional information about the site specificity of phosphorylation of TnI and C-protein was obtained from phoamino acid analysis. TnI and C-protein isolated from control, TPA-, or isoproterenol-stimulated myocytes or phosphorylated in vitro by either PKC or PKA contained phosphate exclusively in phosphoserine. No phosphothreonine was detected for either protein phosphorylated under any of the conditions examined (data not shown).

Effect of Phosphorylation in Situ on Myofibrillar Actomyosin MgATPase Activity Assayed in Vitro—The functional significance of myofibrillar protein phosphorylation in cardiomyocytes was also investigated. Myofibrils were prepared from control, TPA- and isoproterenol-stimulated myocytes and assayed for Ca2+-dependent actomyosin MgATPase activity in vitro. TPA-stimulated phosphorylation was associated with a 25 ± 2% (mean ± S.E.) decrease in maximal activity of myofibrillar MgATPase with no change in the Ca2+ concentration required for activation (Fig. 5). In contrast, myofibrils from isoproterenol-treated cells showed a decreased Ca2+ sensitivity of the enzyme (Ca2+ concentration required for half-maximal stimulation increased from 0.8 μM in control to 3.0 μM in treated cells). No change, however, was observed in maximal MgATPase activity (Fig. 5).

Phosphorylation of Cardiac Myofibrils in Response to α-Adrenergic Stimulation—The potential involvement of PKC-mediated phosphorylation of myofibrillar proteins in hormonal regulation of myocardial cells was also investigated. α-Adrenergic stimulation of cardiomyocytes with 50 μM phenylephrine and 1 μM propranolol (10 min) produced a pattern of myofibrillar protein phosphorylation that was qualitatively and quantitatively similar to that observed with TPA-stimulation (data not shown). TnI and C-protein were stoichiometrically phosphorylated in response to the α-adrenergic agonist, whereas MLC2 was phosphorylated to a lesser extent. Furthermore, phosphorylation of TnI following α-adrenergic stimulation occurred at sites in the protein identical to those phosphorylated in response to TPA or phosphorylated in vitro by PKC (Fig. 6).

DISCUSSION

In this study, we have identified TnI and C-protein as physiological substrates for PKC in intact myocardial cells. Previous studies have shown that TnI (24–26) and C-protein (23) are effective substrates for PKC in vitro. It has been important to confirm that these reactions also occur in situ since protein kinases can phosphorylate proteins in cell-free systems that are not necessarily physiological substrates (44).

FIG. 4. Identification of sites phosphorylated in cardiac C-protein in response to TPA, isoproterenol, and calyculin A (in situ) or by PKC and PKA (in vitro). Myofibrils were phosphorylated in situ in 32P-labeled cardiomyocytes by incubation for 2 h with 32P, followed by a further 10-min incubation with either no addition (control), 100 nM TPA, 100 nM isoproterenol (Isop.), or 100 nM calyculin A (CL-A). Myofibrils were also phosphorylated in vitro by incubation with [γ-32P]ATP and either PKC or PKA. Equal amounts of myofibrillar protein from each condition were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. C-protein was excised from the gel, trypsin digested, and subjected to two-dimensional tryptic phosphopeptide mapping. The origins are indicated by arrows. The radioactivity in each sample was: A, control (456 cpm); B, TPA (1,530 cpm); C, isoproterenol (1,690 cpm); D, calyculin A (487 cpm); E, PKC in vitro (3,360 cpm); F, PKA in vitro (3,492 cpm). Similar results have been obtained in four separate experiments.

FIG. 5. Effects of in situ phosphorylation of myofibrillar proteins on Ca2+-dependent actomyosin MgATPase activity. Isolated cardiomyocytes were preincubated for 2 h (as described in Fig. 2, except that 32P was omitted) followed by a further 10-min incubation with either no addition (control), 100 nM TPA, or 100 nM isoproterenol (Isop.). Myofibrils were then prepared from myocytes, and equal quantities of myofibrillar protein (60 μg) from each condition were assayed for MgATPase activity at various concentrations of calculated free Ca2+. The maximum ATPase activity refers to the total ATPase activity (106 nmol/mg/min) of the control sample assayed at 100 μM free Ca2+ which was taken as 100%. Each point and bar represent the mean ± S.E. for four experiments. Upper panel, control versus TPA. Lower panel, control versus isoproterenol.
Cardiac Myofibrillar Protein Phosphorylation by PKC and PKA

For example, both PKA (45) and the epidermal growth factor receptor tyrosine kinase (46) can phosphorylate isolated myosin light chain from smooth muscle. These kinases do not phosphorylate the light chain of native myosin, however, and the physiological significance of these phosphorylations is doubtful (47). PKC-mediated phosphorylation of cardiac TnI and C-protein does appear to have physiological significance. PKC incorporates phosphate stoichiometrically into TnI and C-protein, not only in native myofibrils, but also in intact myocardial cells stimulated with either phorbol ester or α-adrenergic agonist. Two-dimensional tryptic phosphopeptide mapping shows that the same sites are modified by PKC in vitro and in stimulated cells. Furthermore, phosphorylation has functional significance in that it is reproducibly accompanied by a reduction in maximal activity of myofibrillar actomyosin MgATPase.

Based on the phosphopeptide mapping results presented in this paper (Fig. 3), it is clear that PKC and PKA phosphorylate distinct sites in cardiac TnI. This is consistent with the possibility that phosphorylation by each kinase has different functional effects. Our conclusion, based on phosphopeptide mapping data, differs from the conclusion of Swiderek et al. (48), who have reported that PKC and PKA phosphorylate identical residues in cardiac TnI (Ser-23 and Ser-24 in the bovine sequence which correspond to Ser-22 and Ser-23 in the rat sequence). The reason for this discrepancy is not clear. It is unlikely, however, that it is a result of a species difference since cardiac TnI is a highly conserved protein among mammalian species (27). A further interesting conclusion from the mapping data is that PKA can phosphorylate PKC sites in TnI in vitro. The converse, however, is not true. This suggests that (at least in this specific case) PKC has similar but more rigid sequence recognition requirements for phosphorylation than PKA. Therefore, caution should be exercised when interpreting the results of phosphorylation studies that are based exclusively on in vitro data.

Phosphopeptide mapping and phosphoamino acid analysis of C-protein (Fig. 4) show that (in contrast to what is observed for TnI) TPA and isoproterenol stimulate phosphorylation of identical serine residues in this myofibrillar protein. These results are in agreement with those of Lim et al. (23) who have reported that PKC and PKA phosphorylate the same sites in bovine cardiac C-protein in vitro. Phosphorylation of C-protein and TnI in cardiomyocytes also differs with regard to the rate of phosphate turnover in the two proteins under basal physiological conditions. Treatment of myocytes with calcyculin A, a potent inhibitor of type 1 (IC50 = 2 nM) and type 2A (IC50 = 1 nM) protein phosphatases (43), results in a marked and rapid buildup of phosphate in both PKC and PKA sites of TnI. No increase in phosphate content of C-protein is detected under the same conditions. Phosphate turnover in TnI, therefore, appears to be more rapid than that of C-protein (at least in the absence of hormonal stimulation). Protein phosphatases 1 and 2A, which have both been implicated previously in dephosphorylation of cardiac myofibrillar proteins (49, 50), appear to have a potential regulatory role in determining the state of phosphorylation of TnI.

Both TnT and TnI subunits of tropinin are phosphorylated by PKC in vitro (Fig. 1). However, only TnI is phosphorylated to a significant extent in cardiomyocytes in response to either TPA (Fig. 2) or α-adrenergic stimulation. It is possible that PKC sites in TnT are maximally or near maximally phosphorylated under basal conditions. Of the five myofibrillar proteins that are phosphorylated in myocytes, TnT has the highest basal level of phosphorylation. Furthermore, as is also true for TnI, basal phosphorylation in situ is primarily at sites that are phosphorylated by PKC in vitro. Phosphate in TnT also has a very slow turnover rate as compared with that in TnI. Calyculin A treatment produces a rapid buildup of phosphate in TnI but has no effect on the state of phosphorylation of TnT. Moreover, it is evident that detection of PKC-mediated myofibrillar protein phosphorylation in intact cells may depend on the experimental conditions employed. Previous studies of phosphorylation in intact guinea pig (30) and rabbit hearts (31), for example, have failed to detect PKC-catalyzed phosphorylation of any myofibrillar protein including TnI and C-protein. In the present study, inclusion of the potent phosphatase inhibitor, calyculin A, in all of the isolation buffers may have been crucial in detection of TPA- and α-adrenergic-stimulated phosphorylation of these two proteins.

PKC incorporates phosphate stoichiometrically into four different proteins in native cardiac myofibrils in vitro (Fig. 1). Three of these proteins (C-protein, TnI, and TnT) have been identified previously as substrates for PKC. A fourth substrate for PKC in cardiac myofibrils is identified in this study as MLC2. Smooth muscle and nonmuscle forms of this protein are known to be phosphorylated by PKC at three sites which are distinct from those phosphorylated by myosin light chain kinase (47). Cardiac MLC2, on the other hand, has never been shown to be an effective substrate for PKC either in vitro or in situ. Indeed, there has been general agreement for many years that myosin light chain kinase is exclusively responsible for MLC2 phosphorylation in cardiac cells (1, 2, 12). Disagreement does exist, however, with regard to whether levels of MLC2 phosphorylation are changed during β-adrenergic stimulation of heart (1, 51, 52). In the present study, we have found that MLC2 is consistently phosphorylated to a small extent in response to both TPA and isoproterenol stimulation of cardiomyocytes. We have also recently obtained evidence that MLC2 is phosphorylated in cardiomyo-

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ocytes by both myosin light chain kinase and PKC. 1
PKC-mediated phosphorylation of myofibrillar proteins may have a physiological role in regulation of cardiac actomyosin MgATPase. TPA-stimulated phosphorylation results in a reduced maximal activity of myofibrillar MgATPase with no change in the Ca²⁺ sensitivity, whereas isoproterenol-stimulated phosphorylation has no effect on maximal activity but reduces Ca²⁺ sensitivity (Fig. 5). A Ca²⁺ desensitizing effect of comparable magnitude in response to β-adrenergic stimulation has been shown previously in myofibrils from rabbit heart (8). Because TPA and isoproterenol stimulate phosphorylation of identical sites in C-protein, it is unlikely that this modification has a dominant role in mediating the differential functional effects on the MgATPase. The two different functional effects observed are more likely because of phosphorylation of different sites in TnI by PKC and PKA.

Differential functional effects on the MgATPase. The two

Many different mechanisms operate together, some in concert and others in opposition, to regulate the contractile state of the myocardium. Because of this complexity, it has been difficult to assess the relative contribution of any single mechanism to the overall inotropic responses of the heart to hormonal stimulation. The role of PKA-catalyzed phosphorylation of TnI, for example, is not yet clear. Previous studies have shown that the time course of the positive inotropic response of isolated perfused hearts to β-adrenergic stimulation does not correlate with changes in phosphorylation of TnI (9, 53). Thus, it has been concluded that PKA-mediated phosphorylation of TnI does not have a dominant role in the mechanical response of the mammalian heart to β-adrenergic stimulation. The relative importance of PKC-catalyzed phosphorylation of myofibrillar proteins in mediating the negative inotropic effects of phorbol esters (15, 20, 21) and α-adrenergic agonists (54, 55) on heart cells also remains to be determined. The results of the present investigation, however, demonstrate that PKC-mediated phosphorylation of myofibrillar TnI and C-protein does, indeed, occur in heart cells stimulated by either phorbol ester or α-adrenergic agonists. Furthermore, PKC activation in cardiac cells appears to have functional consequences, not only at the level of Ca²⁺ homeostasis (21, 56) but also at the level of the contractile machinery itself.

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